In addition to the data presented above, invasive cleavage reactions were run with succinate buffer at pH 5.9 in place of the MOPS buffer used in the "standard" reaction; no adverse effects were observed.

The data shown in Figures 42-51 and described above demonstrate that the invasive cleavage reaction can be performed using a wide variety of reaction conditions and is therefore suitable for practice in clinical laboratories.

EXAMPLE 20

Detection Of RNA Targets By Invader-Directed Cleavage

In addition to the clinical need to detect specific DNA sequences for infectious and genetic diseases, there is a need for technologies that can quantitatively detect target nucleic acids that are composed of RNA. For example, a number of viral agents, such as hepatitis C virus (HCV) and human immunodeficiency virus (HIV) have RNA genomic material, the quantitative detection of which can be used as a measure of viral load in a patient sample. Such information can be of critical diagnostic or prognostic value.

Hepatitis C virus (HCV) infection is the predominant cause of post-transfusion non-A, non-B (NANB) hepatitis around the world. In addition, HCV is the major etiologic agent of hepatocellular carcinoma (HCC) and chronic liver disease world wide. The genome of HCV is a small (9.4 kb) RNA molecule. In studies of transmission of HCV by blood transfusion it has been found the presence of HCV antibody, as measured in standard immunological tests, does not always correlate with the infectivity of the sample, while the presence of HCV RNA in a blood sample strongly correlates with infectivity. Conversely, serological tests may remain negative in immunosuppressed infected individuals, while HCV RNA may be easily detected [J.A. Cuthbert (1994) *Clin. Microbiol. Rev.* 7:505].

The need for and the value of developing a probe-based assay for the detection the HCV RNA is clear. The polymerase chain reaction has been used to detect HCV in clinical samples, but the problems associated with carry-over contamination of

samples has been a concern. Direct detection of the viral RNA without the need to perform either reverse transcription or amplification would allow the elimination of several of the points at which existing assays may fail.

The genome of the positive-stranded RNA hepatitis C virus comprises several regions including 5' and 3' noncoding regions (*i.e.*, 5' and 3' untranslated regions) and a polyprotein coding region which encodes the core protein (C), two envelope glycoproteins (E1 and E2/NS1) and six nonstructural glycoproteins (NS2-NS5b). Molecular biological analysis of the HCV genome has showed that some regions of the genome are very highly conserved between isolates, while other regions are fairly rapidly changeable. The 5' noncoding region (NCR) is the most highly conserved region in the HCV. These analyses have allowed these viruses to be divided into six basic genotype groups, and then further classified into over a dozen sub-types [the nomenclature and division of HCV genotypes is evolving; see Altamirano *et al., J. Infect. Dis.* 171:1034 (1995) for a recent classification scheme].

In order to develop a rapid and accurate method of detecting HCV present in infected individuals, the ability of the invader-directed cleavage reaction to detect HCV RNA was examined. Plasmids containing DNA derived from the conserved 5'-untranslated region of six different HCV RNA isolates were used to generate templates for *in vitro* transcription. The HCV sequences contained within these six plasmids represent genotypes 1 (four sub-types represented; 1a, 1b, 1c, and Δ1c), 2, and 3. The nomenclature of the HCV genotypes used herein is that of Simmonds *et al.* [as described in Altamirano *et at., supra*]. The Δ1c subtype was used in the model detection reaction described below.

a) Generation Of Plasmids Containing HCV Sequences

Six DNA fragments derived from HCV were generated by RT-PCR using RNA extracted from serum samples of blood donors; these PCR fragments were a gift of Dr. M. Altamirano (University of British Columbia. Vancouver). These PCR fragments represent HCV sequences derived from HCV genotypes 1a, 1b, 1c, Δ1c, 2c and 3a.

The RNA extraction, reverse transcription and PCR were performed using standard techniques (Altamirano *et al.*, *supra*). Briefly, RNA was extracted from 100 µl of serum using guanidine isothiocyanate, sodium lauryl sarkosate and phenol-chloroform [Inchauspe *et al.*, *Hepatology* 14:595 (1991)]. Reverse transcription was performed according to the manufacturer's instructions using a GeneAmp rTh reverse transcriptase RNA PCR kit (Perkin-Elmer) in the presence of an external antisense primer, HCV342. The sequence of the HCV342 primer is 5'-GGTTTTTCTTTGAGG TTTAG-3' (SEQ ID NO:51). Following termination of the RT reaction, the sense primer HCV7 [5'-GCGACACTCCACCATAGAT-3' (SEQ ID NO:52)] and magnesium were added and a first PCR was performed. Aliquots of the first PCR products were used in a second (nested) PCR in the presence of primers HCV46 [5'-CTGTCTTCACGCAGAAAGC-3' (SEQ ID NO:53)] and HCV308 [5'-GCACGGT CTACGAGACCTC-3' (SEQ ID NO:54)]. The PCRs produced a 281 bp product which corresponds to a conserved 5' noncoding region (NCR) region of HCV between positions -284 and -4 of the HCV genome (Altramirano *et al.*, *supra*).

The six 281 bp PCR fragments were used directly for cloning or they were subjected to an additional amplification step using a 50 µl PCR comprising approximately 100 fmoles of DNA, the HCV46 and HCV308 primers at 0.1 µM, 100 µM of all four dNTPs and 2.5 units of *Taq* DNA polymerase in a buffer containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.1% Tween 20. The PCRs were cycled 25 times at 96°C for 45 sec., 55°C for 45 sec. and 72°C for 1 min. Two microliters of either the original DNA samples or the reamplified PCR products were used for cloning in the linear pT7Blue T-vector (Novagen, Madison,WI) according to manufacturer's protocol. After the PCR products were ligated to the pT7Blue T-vector, the ligation reaction mixture was used to transform competent JM109 cells (Promega). Clones containing the pT7Blue T-vector with an insert were selected by the presence of colonies having a white color on LB plates containing 40 µg/ml X-Gal, 40 µg/ml IPTG and 50 µg/ml ampicillin. Four colonies for each PCR sample were picked and grown overnight in 2 ml LB media containing 50 µg/ml carbenicillin. Plasmid DNA was isolated using the following alkaline miniprep protocol. Cells from